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Human Myeloma Cells Promote the Recruitment of Osteoblast Precursors: Mediation by Interleukin-6 and Soluble Interleukin-6 Receptor*

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ABSTRACT

Multiple myeloma is associated with the development of osteolytic bone disease characterized by a disruption to normal bone resorption and bone formation. Although studies have shown that myeloma cells produce factors that promote bone resorption little data are available examining the mechanism of decreased bone formation or the factors that mediate this effect. In the present study we describe a novel in vitro coculture system in which to investigate the effect of myeloma cells on osteoblast recruitment and differentiation. Under appropriate conditions mesenchymal stem cells were shown to differentiate into colonies of cells, a proportion of which show characteristics of osteoblasts, in that they express alkaline phosphatase activity and stain positively for collagen and calcium. The addition of the human myeloma cells JJN-3, RPMI-8226, or NCI-H929 to these cultures stimulated a significant increase in the total number of colonies ($p < 0.005$) and the proportion of osteoblastic colonies ($p < 0.005$). Media conditioned by these cells also were able to promote the formation of both total and osteoblastic colonies ($p < 0.005$). The addition of an antibody against the interleukin-6 receptor (IL-6R) blocked myeloma cell and myeloma cell–conditioned media induced osteoblast recruitment ($p < 0.01$). Furthermore, media conditioned by myeloma cells incubated with phorbol ester, which promotes IL-6R shedding, or a metalloproteinase inhibitor, which inhibits IL-6R shedding, were able to stimulate ($p < 0.005$) and inhibit osteoblast recruitment ($p < 0.005$), respectively. In addition, soluble IL-6R (sIL-6R) and IL-6 together, but not alone, were able to promote osteoblastic colony formation ($p < 0.01$). Taken together these data show that myeloma cells promote osteoblast recruitment by release of sIL-6R from myeloma cells. (J Bone Miner Res 2000;15:1935–1943)

Key words: multiple myeloma, osteoblast, bone marrow stromal cell, interleukin-6, interleukin-6 receptor

INTRODUCTION

MULTIPLE MYELOMA is a B-cell disease that results in the uncontrolled proliferation of myeloma cells in the bone marrow. A major clinical feature of this disease is the appearance of osteolytic bone lesions characterized by the presence of bone pain, pathological fractures, and the development of hypercalcemia. Although this aspect of the disease is associated with considerable morbidity, our understanding of the mechanism by which bone destruction in multiple myeloma occurs remains incomplete.

Histomorphometric studies, which examine the cellular basis for bone loss, have shown that bone resorption is increased in patients with multiple myeloma.[1–4] This is characterized by a greater proportion of the bone surface

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undergoing bone resorption, an increase in the number of osteoclasts, and an increase in the amount of bone resorbed by these cells.[1–4] This increase in bone resorption is thought to reflect direct induction by the tumor cells, because indices of bone resorption are highest when bone marrow infiltration by tumor cells is greatest.[2,3,5] Under normal circumstances the bone resorbed by osteoclasts, within individual bone remodeling units, is replaced by a quantitatively similar amount of bone by osteoblasts, during the process of bone formation, a process known as "coupling." However, in patients with multiple myeloma there also is evidence for a disruption to the normal process of bone formation. Histomorphometric studies have shown that the bone formation rate ($\mu m^3/\mu m^2$ per day) is increased in patients with multiple myeloma.[2–4] This increase is clearly observed in patients with early myeloma or when the degree of bone marrow plasma cell involvement is modest (5–50% of the mononuclear cell compartment); although, in overt myeloma or when the tumor burden is greater than 50%, the bone formation rate may be similar to that observed in normal subjects or patients with monoclonal gammopathy of undetermined significance.[2,3] These changes in bone formation rate are associated with an increase in the bone surface undergoing formation, as shown by an increase in the surface occupied by osteoblasts and a greater number of osteoblasts per square millimeter and is therefore likely to reflect an increase in osteoblast recruitment.[1–4] However, although osteoblast recruitment is increased the activity of individual osteoblasts appears to be reduced. This is supported by the demonstration that the mineral apposition rate is either unchanged or decreased.[1,3,4] In addition, the wall thickness, a measure of the amount of bone formed within individual remodeling units also is reduced in patients with myeloma.[4]

Thus, histomorphometric analyses have identified characteristic abnormalities in the bone remodeling process in patients with multiple myeloma. These include an increase in bone resorption, an increase in the recruitment of new osteoblasts, particularly in the early phases of the disease, but a failure of these cells to replace completely the bone resorbed. Although maintaining balanced bone formation is essential to prevent rapid bone loss, there are no data investigating the mechanisms of decreased formation or identifying the factors responsible for modulating bone formation in this disease. Therefore, in the present study we have developed a novel in vitro system in which to investigate the effect of myeloma cells on the recruitment and differentiation of osteoblasts. In addition, we show that interleukin-6 (IL-6) and soluble IL-6 receptor (sIL-6R) may mediate the increase in osteoblast recruitment.

MATERIALS AND METHODS

Materials

RPMI-1640 medium, Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (PBS), L-glutamine, trypsin/EDTA, penicillin and streptomycin, MEM nonessential amino acids, sodium pyruvate, Tris, and plasticware were obtained from Gibco Life Technologies, Ltd., (Paisley, U.K.). Alizarin Red S, perchloric acid (70%), and sodium hydroxide were from BDH Chemicals, Ltd. (Poole, UK). Picric acid (98%) and Direct Red 80 were obtained from Aldrich Chemical Co., Ltd. (Gillingham, U.K.). Industrial Methylated Spirit 99 (IMS) was from Hayman, Ltd., (Witham, U.K.). Recombinant human IL-6 (specific activity of 1×10^7 U/mg) was a gift from Sandoz Pharma (Basel, Switzerland). Recombinant human sIL-6R and monoclonal anti-human IL-6R antibody were purchased from R & D Systems (Abingdon, U.K.). BB-94 was a gift from Alan Galloway, British Biotech Pharmaceuticals, Ltd. (Oxford, U.K.). All other chemicals were from Sigma Chemicals Co., (Poole, U.K.).

Culturing human myeloma cells and preparation of myeloma cell–conditioned media

The RPMI-8226 and NCI-H929 human myeloma cell lines were obtained from the European Collection of Animal Cell Cultures (Porton Down, U.K.). The JJN-3 human myeloma cell line was kindly provided by Professor I. Franklin (University of Glasgow, U.K.). The JVM-13 (prolymphocytic leukemia[6]) and Ci-1 (lymphoblastic lymphoma[7]) cell lines were a kind gift from Dr. J. Melo (Imperial College, School of Medicine, London, U.K.). All cell lines were cultured in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and nonessential amino acids and were free from mycoplasma infection. Media conditioned by myeloma cells was prepared by culturing cells at a density of 1×10^6 cells/ml in the presence or absence of a hydroxamate-based metalloproteinase inhibitor BB-94 (20 μM) for 48 h. Supernatants were collected, filtered using a 0.45-μm acrodisk (Gelman Sciences, Northampton, U.K.), and stored at −20°C until required. Media conditioned by myeloma cells stimulated with phorbol 12-myristate 13-acetate (PMA) were prepared by incubating cells at a density of 1×10^6 cells/ml in the presence or absence of PMA (100 nM) for 2 h. The PMA was then removed by pelleting the cells at 100*g* for 10 minutes and resuspending the cells in fresh media and incubating them for a further 48 h. Supernatants were collected, filtered, and stored at −20°C until required.

Preparation of total bone marrow cells

The tibia and femur from 150-g male Wistar rats were removed under aseptic conditions and all adherent tissue was removed. One end of the bone was removed, a hole was made in the opposite end with an 18-gauge syringe needle, and the cells were flushed out with 10 ml DMEM containing 12% FBS, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, 10^{-8} M dexamethasone, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cells were dispersed by repeated pipetting and a single cell suspension was prepared by expelling the cells through a 20-gauge syringe needle.

Establishment of an osteoblast precursor/myeloma cell coculture system

The effect of myeloma cells or media conditioned by myeloma cells or other cell types on osteoblast recruitment was assessed using a modification of a fibroblast colony-forming unit assay described previously.[8,9] Whole bone marrow cells (BMCs; 4×10^4 mononuclear cells/cm^2) were plated in 21 cm^2 or 55 cm^2 petri dishes in DMEM containing 12% FBS, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, 10^{-8} M dexamethasone, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Human myeloma cells (1×10^6 cells/petri dish unless otherwise stated) or media conditioned by myeloma cells (10% of the total culture volume) or other cell types were added immediately. In addition, in some experiments BMCs were cultured in the presence or absence of media conditioned by myeloma cells previously treated with PMA (100 nM) or BB-94 (20 μM). At day 5, the supernatant containing nonadherent BMCs, myeloma cells, conditioned media, or other agents were removed and replaced with fresh media. The media were then changed twice weekly with fresh media that contained no conditioned media, cells, or other agents for a total of 15 days. At the end of the culture period the colonies of cells that formed were analyzed as described below.

Determination of the effect of IL-6 and sIL-6R on osteoblastic recruitment

To investigate the role of IL-6 and the sIL-6R on osteoblast recruitment BMCs were cocultured with either human myeloma cells or media conditioned by either untreated myeloma cells or PMA-stimulated myeloma cells in the presence or absence of monoclonal anti-human sIL-6R antibody or an isotype control antibody (0.2 μg/ml). Antibodies were added at day 1 and day 3 of the culture period. Medium was replaced after 5 days and thereafter twice weekly. In some experiments BMCs were cultured in the presence or absence of IL-6 (100 ng/ml), or sIL-6R (50 ng/ml), or IL-6 and sIL-6R and treated in an identical manner to that described above. At the end of the culture period the colonies were analyzed as described below.

Analysis of osteoblast recruitment

After the culture period the cells were washed with PBS and fixed with IMS for 10 minutes. The cultures were then stained for the presence of colonies expressing alkaline phosphatase activity as described previously.[10] Briefly, cells were incubated in a solution of naphthol phosphate (0.005 mg/ml)/Tris (0.08 M, pH 8.8) containing fast red tr (1 mg/ml) for 30 minutes at room temperature. The cultures were washed under running water, air-dried, and photographed with a digital camera. Colonies were destained with IMS overnight and fixed with 4% formalin. The cultures were then stained with alizarin red (1 mg/ml), pH 6.2, for 10 minutes to show the presence of colonies containing calcium. The plates were then washed under running water, air-dried, and photographed. Subsequently, cultures were demineralized with 5% perchloric acid for 10 minutes and the presence of colonies synthesizing collagen were identified by staining the cultures with direct red (1 mg/ml) in saturated picric acid overnight. The cultures were washed under running tap water, air-dried, and photographed. Finally, the cultures were destained with methanol/0.2M NaOH (50:50, vol/vol) for 10 minutes and the total number of colonies was determined by washing with borate buffer (10 mM, pH 8.8) and staining with methylene blue (1 mg/ml) for 30 minutes. Cultures were then washed under running water, air-dried, and photographed. The acquired images were analyzed using the Bioimage Intelligence Quantifier image analysis program (Bioimage, St. Neots, U.K.) and the total number of each colony type was counted and the number expressed per plate.[11]

Statistical analysis

All experiments were repeated on a minimum of two separate occasions and results are expressed as the mean $\pm$ 1 SD. The n denotes the number of replicate cultures from representative experiments. Comparisons between treatments were performed using a Mann Whitney U test. One-way analysis of variance (ANOVA) was used to examine the relationship between cell numbers and numbers of colonies.

RESULTS

Human myeloma cells promote osteoblast recruitment in vitro

When BMCs were cultured over a period of 15 days in the presence of dexamethasone, ascorbic acid, and β-glycerophosphate, colonies of adherent fibroblasts were formed from the mesenchymal precursor cells present in the cell suspension. A proportion of these colonies express alkaline phosphatase activity and stained positively for calcium and collagen (Fig. 1), which are known characteristics of osteoblasts and osteoblast precursor cells. The addition of increasing numbers of JJN-3 myeloma cells to cultures of BMCs resulted in a significant increase in both alkaline phosphatase, calcium, and collagen positive colonies, and an increase in the total number of colonies ($p < 001$ in each case). This increase reached a maximum with the addition of 1×10^6 cells (Fig. 2). Subsequent studies therefore were undertaken with 1×10^6 myeloma cells. The human myeloma cells RPMI-8226 and NCI-H929 also were able to promote a significant increase in the formation of both osteoblastic and total colonies (Figs. 1 and 3). Coculturing BMCs with JJN-3, RPMI-8226, or NCI-H929 cells promoted a 2.2-, 2.3-, and 2.6-fold increase in the formation of alkaline phosphatase positive colonies, respectively ($p < 0.005$). Myeloma cells also stimulated a significant increase in the formation of colonies that stained for collagen (3.2-fold, 3.5-fold, and 4.3-fold; $p < 0.005$ in each case) and calcium (2.1-fold, 2.9-fold, and 4.5 fold; $p < 0.005$ in each case). The total number of colonies was also significantly increased by each of cell lines examined, with JJN-3, RPMI-8226, and NCI-H929 cells promoting a 2.5-, 2.9-,

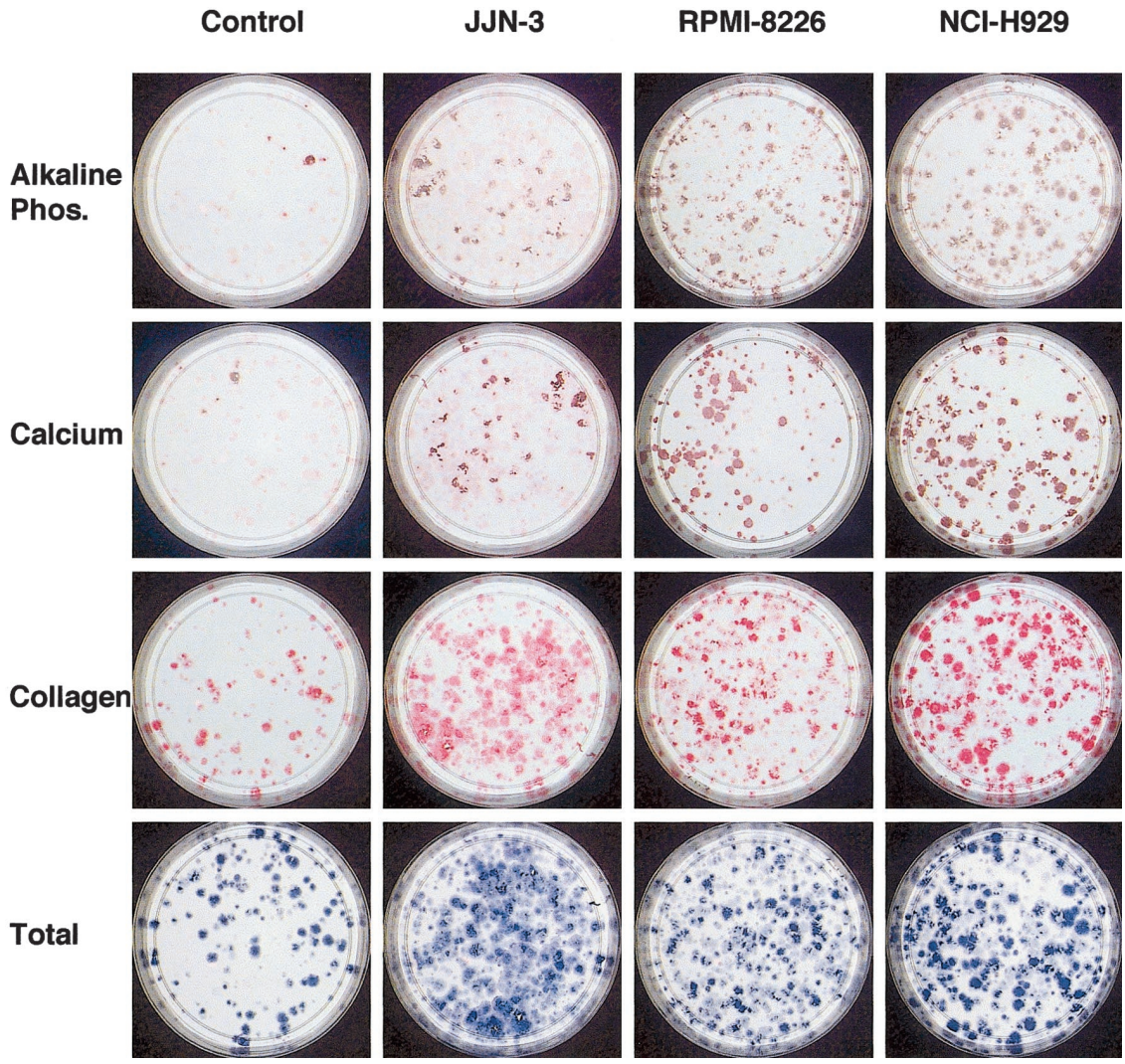

FIG. 1. The effect of human myeloma cells on osteoblastic colony formation. BMCs were cultured either alone or with human myeloma cells. After 15 days, the cultures were stopped and the colonies were stained sequentially for alkaline phosphatase activity, calcium, collagen, and total colonies. The effect of JJN-3, RPMI-8226, and NCI-H929 cells on alkaline phosphatase, calcium, and collagen positive colonies, and total colony formation are shown.

and 2.8-fold increase, respectively ($p < 0.005$, respectively).

The effects on colony formation did not appear to be specific to myeloma cells because when JVM-13 and Ci-1 cells were cocultured with BMCs, there also was a small but significant increase in both osteoblastic colonies and total colonies ($p < 0.005$ in each case) (Fig. 4). However, induction of formation was not as strong as that seen for myeloma cells. Indeed, the numbers of colonies was significantly lower than that promoted by myeloma cells ($p < 0.005$; Fig. 4).

Soluble factors produced by myeloma cells promote osteoblast recruitment in vitro

To determine whether soluble factors released from myeloma cells could stimulate the formation of osteoblastic colonies and/or total number of colonies, BMCs were cultured either alone or in the presence of media conditioned by myeloma cells. Media conditioned by JJN-3, RPMI-8226, or NCI-H929 cells promoted a 1.9-, 2.1-, and 2.0-fold increase in the formation of alkaline phosphatase positive colonies respectively ($p < 0.005$; Fig. 5). Media conditioned by these cells also promoted a significant increase in the formation of colonies that stained positively for collagen (2.1-fold, 3.2-fold, and 3.2-fold; $p < 0.005$ in each case) and calcium (2.0-fold, 3.0-fold, and 2.7-fold; $p < 0.005$ in each case). In addition, JJN-3, RPMI-8226, and NCI-H929 cells also stimulated a 1.8-, 2.5-, and 1.9-fold increase in the total numbers of colonies, respectively ($p < 0.005$; Fig. 5). Media conditioned by the B-cell lines JVM-13 and Ci-1 also were able to stimulate a small increase in colony number ($p < 0.005$ in each case) although this was significantly

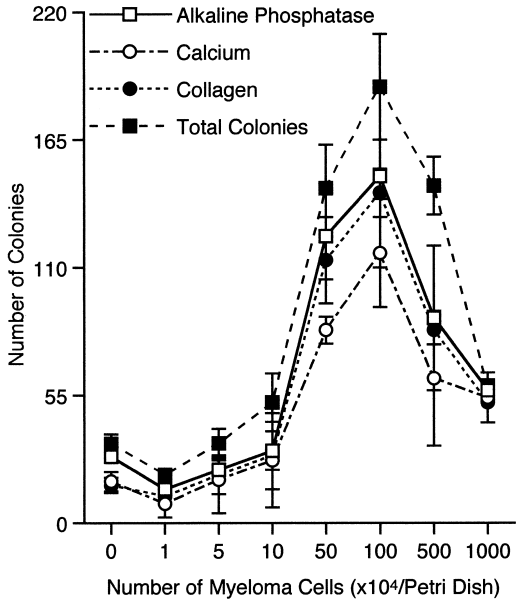

FIG. 2. The effect of JJN-3 human myeloma cells on the formation of alkaline phosphatase, calcium, and collagen positive colonies and the total numbers of colonies. BMCs were cultured either alone or with increasing numbers of JJN-3 cells. After the first 5 days of the culture period, myeloma cells were removed and replaced with fresh media and thereafter the media was replaced twice weekly. After 15 days, cultures were stained sequentially for alkaline phosphatase activity, calcium, collagen, and total colonies. Data represent the mean ± 1 SD ($n = 3$) of a representative experiment. $p < 0.001$ BMCs cultured with myeloma cells compared with BMCs cultured in the absence of myeloma cells (ANOVA).

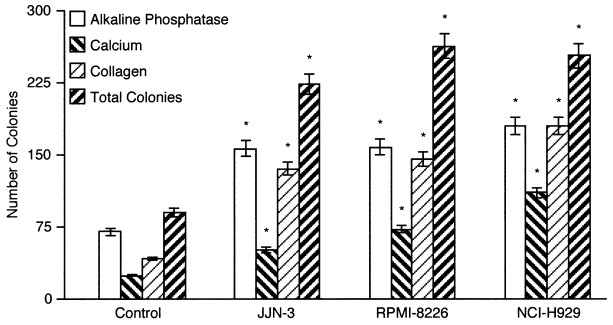

FIG. 3. The effect of the human myeloma cells, JJN-3, RPMI-8226, and NCI-H929, on the formation of alkaline phosphatase, calcium, and collagen positive colonies and the total numbers of colonies. BMCs were cultured either alone or with the human myeloma cell lines JJN-3, RPMI-8226, or NCI-H929. After the first 5 days of the culture period, myeloma cells were removed and replaced with fresh media and thereafter the media was replaced twice weekly. After 15 days, cultures were stained sequentially for alkaline phosphatase activity, calcium, collagen, and with methylene blue to identify the total number of colonies. Data represent the mean ± 1 SD ($n = 6$) of a representative experiment. *$p < 0.005$ compared with BMCs cultured in the absence of myeloma cells (control).

lower than that observed with myeloma cell–conditioned media ($p < 0.005$ in each case).

sIL-6R released from myeloma cells stimulates osteoblast recruitment

Media conditioned by myeloma cells can promote the formation of osteoblastic colonies and total number of colonies; however, the identity of the factor or factors responsible for mediating this is unknown. Recently, we have shown that human myeloma cells shed the IL-6R from the cell surface.[12] We therefore investigated whether the effect of myeloma cells on osteoblastic colony formation could be mediated by the presence of sIL-6R in the medium. Because PMA increases shedding of sIL-6R from myeloma cells (300% increase from JJN-3 and RPMI-8226 cells and 50% increase from NCI-H929 cells),[12] we first investigated whether media conditioned by myeloma cells treated with PMA could increase osteoblastic colony formation. Indeed, conditioned media from PMA-treated JJN-3, RPMI-8226, and NCI-H929 cells promoted a 17, 44, and 53% increase in the formation of alkaline phosphatase positive colonies, when compared with BMCs cultured with media conditioned by appropriate unstimulated myeloma cells ($p < 0.005$, respectively; Table 1). PMA-treated–conditioned media from each of these cells also promoted a significant increase in the colonies that stained positively for collagen and calcium (JJN-3, 58% and 50%; RPMI-8226, 47% and 57%; and NCI-H929 31% and 47%, respectively; $p < 0.005$ in each case). In addition, conditioned media from PMA-treated JJN-3, RPMI-8226, and NCI-H929 cells also promoted a 47, 51, and 49% increase in the formation of the total number colonies ($p < 0.005$, respectively [Table 1]). In contrast, when BMCs were incubated with media conditioned by myeloma cells treated with the hydroxamate-based metalloproteinase inhibitor, BB-94, which has previously been shown to inhibit release of IL-6R (by 60% from RPMI-8226 cells),[12] there was a significant decrease in the number of osteoblastic and total colonies. Conditioned media from BB-94– treated JJN-3, RPMI-8226, and NCI-H929 cells caused a 54, 42, and 38% decrease in alkaline phosphatase positive colonies ($p < 0.005$; Table 1), a 51, 45, and 45% decrease in collagen positive colonies ($p < 0.005$), a 51, 62, and 51% decrease in calcium positive colonies ($p < 0.005$), and a 49, 53, and 37% decrease in total colony number, respectively ($p < 0.005$; Table 1). BB-94 had no effect on osteoblastic or total colony formation when added directly to BMC cultures.

Although PMA stimulated and BB-94 inhibited the formation of osteoblastic and total colonies, the nature of the soluble mediator remains unclear because these agents could modulate production of factors other than sIL-6R. To determine whether sIL-6R could mediate these responses, cultures were established in the presence or absence of an inhibitory monoclonal human anti–sIL-6R antibody (0.2

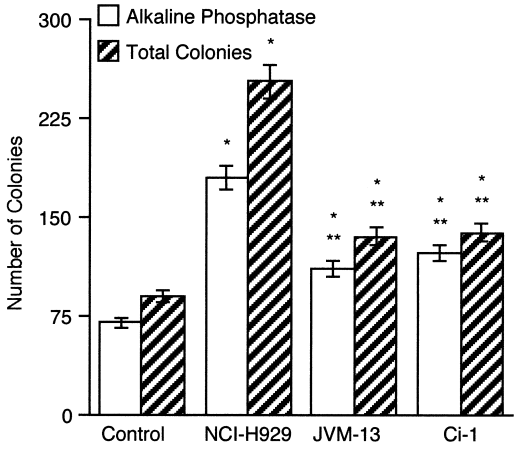

FIG. 4. The effect of human myeloma cells and JVM-13 prolymphocytic leukemia and Ci-1 lymphoblastic lymphoma cells on the formation of alkaline phosphatase positive and total numbers of colonies. BMCs were cultured either alone or with either NCI-H929 myeloma cells or JVM-13 or Ci-1 cells. After the first 5 days of the culture period, cells were removed and replaced with fresh media and thereafter the media was replaced twice weekly. After 15 days, cultures were stained for alkaline phosphatase activity and with methylene blue to identify the total number of colonies. Data represent the mean ± 1 SD of a representative experiment. $n = 6$ except in cultures of BMCs incubated with B cells in which $n = 4$. *$p < 0.005$ compared with BMCs cultured in the absence of myeloma cells or B cells (control). **$p < 0.005$ compared with BMCs cultured with NCI-H929 myeloma cells.

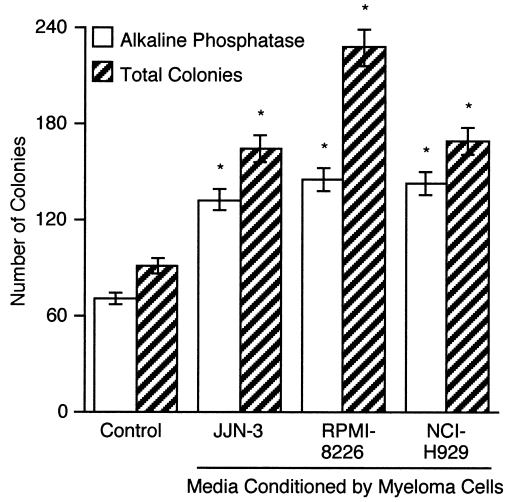

FIG. 5. The effect of soluble mediators released from myeloma cells on the formation of alkaline phosphatase positive and total numbers of colonies. BMCs were cultured either alone or with media conditioned by JJN-3, RPMI-8226, or NCI-H929 myeloma cells. After the first 5 days of the culture period, nonadherent cells and conditioned media were removed and replaced with fresh media. After 15 days, cultures were stained for alkaline phosphatase activity and with methylene blue to identify the total number of colonies. Data represent the mean ± 1 SD ($n = 6$) of a representative experiment. *$p < 0.005$ compared with BMCs cultured in the absence of media conditioned by myeloma cells (control).

μg/ml) or an isotype control antibody (0.2 μg/ml). The anti–sIL-6R antibody, in the absence of conditioned media, had no effect on colony formation. However, the anti–sIL-6R antibody was able to cause a significant decrease in the number of alkaline phosphatase positive (Table 2), collagen positive, and calcium positive (data not shown) and total number of colonies (Table 2) induced by JJN-3, RPMI-8226, and NCI-H929 cells ($p < 0.01$ in each case). In addition, the anti–sIL-6R antibody also prevented the increase in alkaline phosphatase positive (Table 2), collagen positive and calcium positive (data not shown), and total numbers of colonies (Table 2) induced by media conditioned by both myeloma cells and PMA-stimulated myeloma cells ($p < 0.01$ in each case).

sIL-6R and IL-6 promote the recruitment of osteoblastic cells in vitro

Because we have shown that inhibiting release of sIL-6R from myeloma cells and inhibitory anti–sIL-6R antibodies block the induction of alkaline phosphatase positive and total numbers of colonies, we have investigated whether exogenous sIL-6R and/or IL-6 could promote these effects. BMCs were cultured either alone or in the presence of IL-6 (100 ng/ml), sIL-6R (50 ng/ml), or IL-6 plus sIL-6R. IL-6 and sIL-6R alone were unable to promote the formation of alkaline phosphatase positive and total colonies (Fig. 6). However, when both IL-6 and sIL-6R were added to the cultures together, a 3-fold increase in both alkaline phosphatase positive ($p < 0.01$) and total numbers of colonies was observed ($p < 0.01$; Fig. 6).

DISCUSSION

The osteolytic bone disease that develops in patients with multiple myeloma is characterized by an increase in bone resorption and a decrease in bone formation. Studies have shown that myeloma cells can produce a number of factors that promote bone resorption,[13–19] although the specific identity of the factor(s) responsible in vivo remain unclear. However, no data are available investigating the factors responsible for modulating bone formation. In the present study we have developed a novel coculture system in which to investigate the mechanism by which myeloma cells affect bone formation and to identify the factors involved. Under appropriate conditions, mesenchymal stem cells present in the bone marrow differentiate into colonies of cells, a proportion of which show characteristics of osteoblasts. These include the expression of alkaline phosphatase activity, the synthesis of collagen, and the ability to calcify. The addition

TABLE 1. THE EFFECT OF PMA AND BB-94 TREATMENT OF MYELOMA CELLS ON THE FORMATION OF ALKALINE PHOSPHATASE AND TOTAL NUMBERS OF COLONIES

Treatment[a]	*Number of colonies*[b]	
	Alkaline phosphatase	*Total*
BMC cultured alone	69.8 ± 6.6	90.5 ± 8.6
BMC cultured with		
CM from JJN-3 cells	131.5 ± 7.0*	164.1 ± 10.3*
CM from PMA-stimulated JJN-3 cells	153.3 ± 9.0†	240.6 ± 14.0†
CM from BB-94–treated JJN-3 cells	60.7 ± 5.0†	84.5 ± 9.6†
BMC cultured with		
CM from RPMI-8226 cells	144.1 ± 3.5*	227.1 ± 9.2*
CM from PMA-stimulated RPMI-8226 cells	207.8 ± 13.0†	342.0 ± 16.4†
CM from BB-94–treated RPMI-8226 cells	83.5 ± 14.9†	108.0 ± 3.7†
BMC cultured with		
CM from NCI-H929 cells	142.6 ± 14.0*	168.3 ± 13.7*
CM from PMA-stimulated NCI-H929 cells	217.6 ± 12.2†	251.0 ± 13.3†
CM from BB-94–treated NCI-H929 cells	90.7 ± 8.7†	104.7 ± 9.9†

CM, conditioned media.

[a] BMCs were cultured either alone or with CM from myeloma cells or CM from PMA (100 nM) or BB-94 (20 μM)–treated myeloma cells.

[b] Results are expressed as the mean ± 1 SD ($n = 6$ except in cultures of BMC treated with CM from BB-94–treated myeloma cells where $n = 4$).

* $p < 0.005$ compared with BMCs cultured alone.

† $p < 0.005$ compared with BMCs cultured with untreated CM from the appropriate myeloma cells.

TABLE 2. THE EFFECT OF MONOCLONAL ANTI-HUMAN sIL-6R ANTIBODY ON MYELOMA CELL INDUCED ALKALINE PHOSPHATASE AND TOTAL COLONY FORMATION

Treatment[a]	*Number of colonies*[b]			
	Alkaline phosphatase		*Total*	
	Control	*Anti–sIL-6R*	*Control*	*Anti–sIL-6R*
BMCs cultured with				
JJN-3 cells	148.2 ± 4.3	89.7 ± 7.5*	230.2 ± 8.3	110.0 ± 2.2*
CM from JJN-3 cells	125.5 ± 6.5	70.0 ± 10.5*	159.5 ± 7.5	89.0 ± 7.3*
CM from PMA-treated JJN-3 cells	149.7 ± 4.6	108.7 ± 5.0*	236.7 ± 8.4	157.7 ± 12.7*
BMCs cultured with				
RPMI-8226 cells	154.2 ± 7.2	82.0 ± 3.2*	257.2 ± 14.7	151.2 ± 12.6*
CM from RPMI-8226 cells	149.2 ± 4.8	94.0 ± 4.2*	215.7 ± 10.6	121.5 ± 9.5*
CM from PMA-treated RPMI-8226 cells	203.7 ± 16.8	140.2 ± 19.4*	317.0 ± 11.9	210.7 ± 12.0*
BMCs cultured with				
NCI-H929 cells	194.7 ± 17.4	95.2 ± 3.6*	268.2 ± 15.4	148.0 ± 6.9*
CM from NCI-H929 cells	141.2 ± 7.8	93.0 ± 10.0*	160.0 ± 5.7	124.7 ± 10.0*
CM from PMA treated NCI-H929 cells	209.0 ± 37.4	145.0 ± 4.7*	263.0 ± 5.1	175.5 ± 7.0*

[a] BMCs were cultured with either myeloma cells, or media conditioned by myeloma cells, or media conditioned by myeloma cells stimulated with PMA in the presence of either monoclonal anti-human sIL-6R antibody (0.2 μg/ml) or isotype control antibody (0.2 μg/ml).

[b] Results are expressed as the mean ± 1 SD ($n = 4$).

* $p < 0.01$ compared with appropriate cultures in the presence of isotype control antibody.

of the human myeloma cells JJN-3, RPMI-8226, and NCI-H929 to these cultures of BMCs resulted in a significant increase in the total number of colonies that form and the number of colonies that express alkaline phosphatase activity and stain positively for collagen and calcium. Furthermore, when media conditioned by myeloma cells was added to cultures of BMCs this was also able to stimulate an increase in the total number of colonies and those exhibiting an osteoblastic phenotype. This effect on osteoblastic and total colony formation appears to be restricted to myeloma cells because although alternative cell types (JVM-13 and Ci-1) also promoted an increase in the formation of colonies, the magnitude of stimulation was small in comparison with the response elicited by myeloma cells.

The increase in the number of osteoblastic colonies and therefore the recruitment of osteoblastic precursors is con-

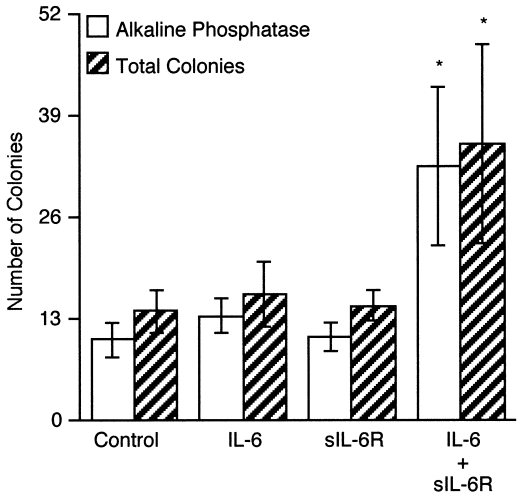

FIG. 6. The effect of sIL-6R and IL-6 on the formation of alkaline phosphatase positive and total numbers of colonies. BMCs were cultured in 21-cm^2 petri dishes either alone or in the presence of IL-6 (100 ng/ml), sIL-6R (50 ng/ml), or IL-6 (100 ng/ml) together with sIL-6R (50 ng/ml). IL-6 and sIL-6R were added on day 1 of cultures only. After the first 5 days of the culture period, media containing cytokines were removed and replaced with fresh media. After 15 days, cultures were stained for alkaline phosphatase activity and with methylene blue to identify the total number of colonies determined. Data represent the mean ± 1 SD ($n = 4$) of a representative experiment. $p < 0.01$ compared with BMC cultured either alone or with IL-6 or sIL-6R.

sistent with histomorphometric studies, which have shown that the proportion of bone surfaces occupied by osteoblasts and the number of osteoblasts per unit area are increased in patients with myeloma.[1–4] This suggests that this coculture system will prove to be a valuable in vitro model for investigating the effects of myeloma cells on osteoblast recruitment. In addition, this system may also allow us to investigate the effect on the differentiation of osteoblasts. Histomorphometric studies suggest that the activity of osteoblasts is reduced in patients with myeloma and this is likely to account for the increased negative remodeling balance and rapid bone loss. This decrease in activity is likely to reflect an effect of myeloma cells on the differentiation of osteoblasts and/or on the function of the mature cells.

The addition of an inhibitory antibody specific for the human IL-6R, to the culture system, also was shown to inhibit osteoblastic colony formation, strongly suggesting that the increase in osteoblast recruitment was mediated by the IL-6R. The IL-6R exists in two forms, a membrane-bound form[20] and soluble form,[21] both of which can bind IL-6 and associate with gp130,[22] the signal transducing component of the IL-6R complex.[23] Because the anti–IL-6R antibody will bind to both forms of the IL-6R these studies are unable to distinguish between an effect on the soluble form and/or the membrane-bound form of the receptor. However, media conditioned by myeloma cells treated with PMA, which promotes IL-6R shedding and therefore increases the concentration of sIL-6R in the media,[12] promoted the recruitment of osteoblast precursors. Conversely, treatment with a metalloproteinase inhibitor, which is known to prevent IL-6R shedding,[12] decreased colony formation. Furthermore, when sIL-6R was added in conjunction with IL-6 to BMC cultures there was strong induction of osteoblast recruitment. However, when these factors were added independently, there was no increase in colony formation. Taken together these data suggest that sIL-6R released from myeloma cells is important in promoting osteoblast recruitment. Because we have shown previously that the myeloma cell lines used in this study do not produce IL-6 but that BMCs and osteoblasts can produce this cytokine[24] and that these cells can be stimulated to produced increased concentrations of IL-6 when cocultured with myeloma cells,[24] it is likely that IL-6R is shed from the surface of myeloma cells and this binds to IL-6 released by the BMCs. It is this complex that may then be able to associate with gp130 on the surface of osteoblast precursors to promote their recruitment and differentiation. Although our data strongly suggest that sIL-6R, released from myeloma cells, plays an important role in this process we cannot rule out the possibility that other factors may also play a minor role because the anti–sIL-6R antibody was unable to inhibit completely colony formation induced by media conditioned by PMA-treated myeloma cells. Interestingly, this complex of IL-6 and sIL-6R has also been shown to be able promote osteoclast formation in vitro, raising the possibility that these agents may also be able to promote bone resorption in vivo.[25]

The data presented in this study describe the development of a coculture system in which myeloma cells are maintained with BMCs and modulate their recruitment and differentiation into colonies of cells of the osteoblast lineage. In this system we have shown that myeloma cells promote the recruitment of osteoblast precursors, which is consistent with histomorphometric data, and show that this effect may be mediated by the sIL-6R released from myeloma cells.

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